“What a difference a buffer can make!” Optimizing and improving crystallization methods of a protease with the aid of biophysics and automation

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Abstract

Protein disorder can plague protein crystallography where the first important goal is to identify conditions that grow stable, ordered, and well diffracting crystals. Initial crystal trials for protease X produced numerous crystals in a myriad of conditions; many of which did not produce well diffracting crystals. Optimizing all of the most obvious parameters: pH, temperature, salt and precipitant concentration continued to produce poor crystals. The fast growth rate and consistently poor diffraction gave an indication that the protein may have some stability issues that could stem from the choice of storage buffer. Recent studies have shown that incorporating thermal melting (Tm) assays into the crystallization screening experiments completely opens up a greater opportunity to explore the protein’s stability prior to crystal trials. This poster will look at the use of thermal shift and the use of automation to screen, characterize and optimize poorly diffracting crystals to well ordered crystals diffracting to beyond 2 angstrom resolution.

Introduction

Thermal, chemical and melting properties play a major role in choosing a method for crystal production. Three methods were investigated with the accompaniment of three pieces of equipment that were incorporated or designed to aid high through-put protein crystallography. Protease X was used in this experimental study with the intention of producing an ordered crystal for structural determination but the crystals that were produced displayed a major diffraction-limiting disorder. The dominant disorder is likely due to conformation variations, chemical microheterogeneity, small molecule displacements and rotations from site to site within the crystal, and perhaps also macromolecular impurities when present at very large concentrations. Ultimately, these issues are addressed through observing the behavior and stability of the protein with thermal melting assays and making the proper changes necessary to improve the diffraction quality.

The conceptual basis for thermal melting (Tm) is that folded and unfolded proteins can be distinguished through exposure to a hydrophobic fluoroprobe. Thermally induced unfolding is an irreversible unfolding process following a typical two-state model with a sharp transition between the folded and unfolded states, where *Tm* is defined as the midpoint of temperatures of the protein-unfolding transition. When a protein starts to unfold or melt, the dye binds to exposed hydrophobic parts of the protein, resulting in a significant increase in fluorescence emission. A positive *Tm* can be coupled to an increase in structural order and a reduced conformational flexibility, whereas a decrease in stability, negative *Tm*, indicates that the buffer induces protein structural changes toward a more disordered conformation or it can be a sign of misfolding. When no measurable transitions occur, it could be possibly caused by destabilization/partial unfolding and potentially aggregation of the proteins. *Tm* shifts larger than 2 degrees is considered to be significant.

Objectives

- Crystallize protease X using vapor diffusion methods
- Display how optimizing crystals using automation can aid faster turnaround time
- Implement thermal shift assays to help identify conditions that will improve protein stability
- Incorporate results from thermal melting assays into protein before executing crystallization trials

Materials and Methods

At 23 mg/ml, protease X was incubated with 3 mM peptide for 24 hours at 4°C (storage buffer: 25 mM Hepes pH 7.5, 300 mM NaCl, 10 mM DTT and 1 mM EDTA). Crystallization trials were carried out using vapor diffusion in 96-well hanging drops that ranged from 400-600 nano-liters total volume at 20°C. Hampton Index HT, Crystal HT, Qiagen Peg and pH clear suites are the commercial screens that were consistently used throughout the screening process to initiate crystal growth. All screens were dispensed by the TTP Mosquito, which is a low-volume liquid handler that accurately aliquots and dispenses a 96-well screen in less than 3 minutes with very little protein. Most physical observations were monitored through the use of the Formulatrix Rockimager. The imager provides user-friendly software that allows plates to be imaged, viewed, maintained and scored over a specific course of time. This imaging schedule was designed to produce images of each plate over a course of 5 weeks with 10 different time-stamps. Optimization was done manually and with the aid of the Formulatrix Formulator which dispenses ingredients into a SBS formatted plate through software that has been integrated with the Formulatrix imager. This piece of equipment allows for many different formulations of ingredients to be accurately dispensed into a plate in a timely manner.

Thermal Shift Assays

The protein was diluted to 0.1 mg/ml with buffer: 25 mM HEPES, pH 7.5, 300 mM NaCl, 10 mM DTT, 1 mM EDTA and 1:1000 dilution of sypro orange was added to this solution. This sample was then set-up in an ABI Prism 96 well optical reaction plate by Applied Biosystems in triplicate as a control in parallel with buffer without protein within the same plate then sealed with microAmp-optical adhesive film by Applied Biosystems. A Real-Time PCR machine (7900HT) was used to heat the samples from 25°C -95°C in increments of 2°C. Tm was established using the Boltzmann model used to fit the fluorescence imaging data . The simplest way to calculate Tm values is to determine the maximum of the first derivative; this feature is offered by most PCR software packages.

Buffer Exchange

Zebra Micro Spin Desalting Columns were used as an alternative to dialysis buttons for buffer exchange. 10% 1.6 M sodium citrate tribasic was added to the storage buffer through a very simple protocol that requires centrifugation of the new buffer through the column 4X before eluting the protein in the final spin.

Results

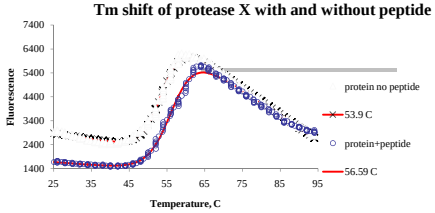
Crystals began to grow within an hour and produced massive showers of crystals for several conditions. Optimization of four different conditions using an increased drop volume with two ratios enabled the production of larger crystals (200 micrometers X 50 micrometers). Theses crystals were harvested after a 2 day incubation using 10-30% glycerol as the cryo-protectant (later changed to 5-30% ethylene glycol for most crystals because the diffraction quality improved 4 Å -fold). None of the crystals diffracted better than 7.5 Å X 10 Å (anisotropic). Over time the construct and crystal conditions changed because they performed better in assay development for this protein but the diffraction was only minimally improved (best diffraction: 3.2 Å X 4 Å still anisotropic).

Tm for protease X was determined to be ~53°C without peptide and 56°C with peptide. This assay was repeated with protease + peptide only using 10% of 2 commercial screens in 2 separate 96-well PCR plates. Conditions that produced a thermal shift 3°C or more were deemed as significant and a potential candidates for buffer exchange. Four conditions were selected to repeat in triplicate with and without protein to observe the condition’s interaction with the buffer in the presence of sypro orange. One reagent consistently appeared to shift in all of the screens with different concentrations. Sodium citrate tribasic was the dominating factor that influenced the Tm and became the additive for the new buffer to utilize in the next round of crystallization trials.

0.2 M Potassium thiocyanate 15%Peg3350 (2:1)

0.2 M Potassium thiocyanate 18%Peg3350 (1:1)

Tm shift of protease X with and without peptide



Raw Tm data of protease X comparison of 2 conditions

Protease X	1	2	3	10	11	12
A-protein (no reagents & no peptide)	53.9	54.4	54	52.3	51.8	52.3
B-reagents (storage buffer + sypro no protein)	28.5	40.7	37.2	30	38.5	37.8
C-protein (no peptide) 10% reagent	54.2	55.2	53.6	55.2	56	55.4
D-protein (plus peptide) 10% reagent	56.7	56.9	56.8	56.4	55.6	55.8
E-protein (plus peptide) no reagent	56.6	56.5	56.5	55.8	56.2	56.4
Reagents	0.01 M Hepes pH 7.5, 0.14 M tri-sodium citrate			0.16 M tri-sodium citrate		

A second round of crystal trials with protease X at 23 mg/ml in the new buffer produced crystals that grew at a slower rate with a slightly different morphology in a 96-well hanging drop. One condition appeared to be more interesting than the rest and was later chosen as the condition to be optimized into larger drops (0.2-1 M ammonium citrate dibasic, 13-19% Peg3350 and one row contained 5 mM DTT). Optimizing the crystals in sitting drops plates with multiple daughter wells enabled the protein to be set up in 2 ratios (1:1 and 2:1). Crystals began to grow from the 24-well sitting drop plate that grew in the presence of 5 mM DTT only. Nucleation became apparent after 3-5 days and continued up to 14 days later for some drops. Three crystals were harvested (cryo-protectant: 5-30% ethylene glycol) and shipped to the Synchrotron for data collection. All of the crystals diffracted 1.9-3 Å .

17% Peg3350
0.2 M ammonium citrate dibasic
5 mM DTT

13% Peg3350
0.2 M ammonium citrate dibasic
5 mM DTT

Conclusion

These experiments demonstrate the power of using biophysics in concert with crystallization methods. Using the two methods exposes a better understanding of the relation between protein stability and how it can impact disorder and diffraction and ultimately provide an important guide in attempts to produce high-quality crystals. The outcome for these experiments has provided some insight and forethought as to how difficult crystal systems can be approached then optimized in effort to obtain high ordered crystals.

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